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## Development and Characterization of a Subtilin-Regulated Expression System in *Bacillus subtilis*: Strict Control of Gene Expression by Addition of Subtilin

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**A system for subtilin-regulated gene expression (SURE) in *Bacillus subtilis* that is based on the regulatory module involved in cell-density-dependent control of the production of subtilin is described. An integration vector for introduction of the essential sensor-regulator couple *spaRK* into the *amyE* locus of the *B. subtilis* chromosome and a *B. subtilis* 168-derived production host in which the *spaRK* genes were functionally introduced were constructed. Furthermore, several expression plasmids harboring the subtilin-inducible wild-type *spaS* promoter or a mutated derivative of this promoter were constructed, which facilitated both transcriptional and translational promoter-gene fusions. Functional characterization of both *spaS* promoters and the cognate expression host could be performed by controlled overproduction of the  $\beta$ -glucuronidase (GusA) and green fluorescent protein (GFP) reporters. Both *spaS* promoters exhibited very low levels of basal expression, while extremely high levels of expression were observed upon induction with subtilin. Moreover, the level of expression depended directly on the amount of inducer (subtilin) used. The wild-type *spaS* promoter appeared to be more strictly controlled by the addition of subtilin, while the highest levels of expression were obtained when the mutated *spaS* promoter was used. Induction by subtilin led to 110- and 80-fold increases in GusA activity for the *spaS* promoter and its mutant derivative, respectively. Since the SURE system has attractive functional characteristics, including promoter silence under noninducing conditions and a controlled and high level of expression upon induction, and since it is not subject to catabolite control, we anticipate that it can provide a suitable expression system for various scientific and industrial applications.**

*Bacillus subtilis* is generally considered to have great industrial potential for production and secretion of proteins of clinical interest, like interferon (37), insulin (36), pathogenic antigens (1), and toxins (45), or enzymes of great industrial interest, like proteases (17),  $\alpha$ -amylase (18), and lipases (17). The major advantages of *B. subtilis* compared to other host production systems are high-cell-density growth and secretion of the synthesized protein into the cultivation medium, which facilitates isolation and purification of the protein during downstream processing (5, 31).

In order to produce homologous or heterologous proteins, several systems for inducible gene expression in *B. subtilis* have been developed. The starch-inducible amylase promoter is frequently used for production of heterologous proteins in which the desired protein is fused to the  $\alpha$ -amylase promoter and leader peptide, which efficiently drives secretion of the protein produced into the culture medium (1, 17). Several prophage-derived heat-inducible gene expression systems that show very tight control of gene expression have been described; however, the levels of expression upon maximum induction are relatively low compared to those of other inducible gene expression

systems (7, 17, 31). An inducible gene expression system based on the regulation machinery of *Escherichia coli* Tn10-encoded tetracycline resistance has been shown to be functional in *B. subtilis* (14). This system has been reported to generate 100-fold-increased expression upon induction with tetracycline; however, considerable basal levels of expression are observed. A more tightly regulated variant of this system has been developed, but it appeared to generate lower maximal levels of expression upon induction (14). Furthermore, the well-known *E. coli lac* repressor-based expression system has been functionally implemented in *B. subtilis* using a two-plasmid system, which allowed isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-controlled gene expression in the latter species. This system was reported to exhibit no expression without addition of the inducer, while very high levels of expression (10 to 15% of the total protein) were observed after IPTG induction (30). This control mechanism is also used in an expression system that employs the hybrid P<sub>spac</sub> promoter, which is composed of the *Bacillus licheniformis* penicillinase promoter and the *E. coli lac* operator, in which IPTG-mediated derepression leads to transcription activation and yields high levels of gene expression (49). Finally, the *xylA* system, in which a gene of interest is fused to the xylose-inducible *xylA* promoter and is integrated into the *amyE* locus of the *B. subtilis* chromosome, has been reported to generate very high transcription activity upon xylose induction, whereas the basal level of expression is low (2,

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20). A direct comparison of the  $P_{spac}$ - and  $P_{xylA}$ -inducible promoter systems has been described (2). The results suggest that the *xylA* promoter exhibits lower basal levels of expression than the *spac* promoter, while it generates higher levels of expression following induction. However, regulation of both  $P_{xylA}$  and  $P_{spac}$  is subject to glucose repression, which results in lower levels of expression and tighter regulation in glucose-containing culture media. As a consequence, high protein production levels can be achieved only by using media with extremely low glucose levels, which in turn increases "promoter leakage," which can be a problem when the gene product of interest has detrimental effects on the producer.

In another gram-positive host, *Lactococcus lactis*, a well-characterized inducible expression system, the so-called nisin-controlled gene expression (NICE) system, has been described, and this system exploits the autoregulatory characteristics of the production of the lantibiotic nisin by this bacterium for controlled expression of homologous and heterologous genes that can be triggered by addition of the inducer nisin (10, 25, 27). The NICE system now includes several *nisRK*-expressing production hosts and a variety of convenient expression vectors that allow translational or transcriptional fusion of the gene of interest to the tightly controlled *nisA* promoter (35). The NICE system has been used successfully in many metabolic engineering studies and for the construction of nisin-controlled autolytic lactococcal strains and several conditional mutants, as well as in several industrial-scale protein and peptide production approaches (23). Furthermore, use of a dual plasmid-based cassette system or derivatives of this system has shown that the NICE system could be functionally implemented in a wide range of alternative gram-positive hosts, including *B. subtilis* (12).

*B. subtilis* ATCC 6633 produces the lantibiotic subtilin, which exhibits a high level of homology to the well-known lantibiotic nisin in terms of the primary sequence, secondary and tertiary structural features, and the mechanism of production and regulation (23, 41). Similar to nisin production in *L. lactis*, the production of subtilin in *B. subtilis* is subject to quorum-sensing control that depends on sensing of subtilin by a dedicated sensor histidine kinase (SpaK) and subsequent signal transduction to the corresponding response regulator (SpaR). Upon phosphorylation, SpaR binds to so-called *spa* boxes in the promoter regions upstream of the *spaB*, *spaI*, and *spaS* genes located in the subtilin biosynthesis gene cluster, thereby triggering promoter activation (22, 43, 44). Additionally, production of subtilin in *B. subtilis* has been shown to be dually controlled since *spaRK* expression is controlled by the transition-state regulator sigma H (43). As a consequence of this mode of regulation, the level of production of subtilin during the early and mid-log phases of growth is relatively low, while high levels of subtilin are produced during the late exponential and transition-state growth phases (21, 43).

Here we describe the construction of a subtilin-regulated gene expression (SURE) system for *B. subtilis* in which *spaR*- and *spaK*-dependent signal transduction is used to control  $P_{spaS}$ -driven gene expression. Construction of a plasmid for integration of the required regulatory factors encoded by *spaRK*, as well as several multicopy expression vectors harboring *spaS* promoter-derived subtilin-responsive promoter elements, is described. The SURE system's functionality is exemplified by efficient and controlled overproduction of the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference
<b>Strains</b>		
<i>E. coli</i> MC1061	F <sup>-</sup> <i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7696 $\Delta$ ( <i>lac</i> )X74 <i>galU galK hsdR2 mcrA mcrB1 rspL</i>	48
<i>L. lactis</i> MG1363	Plasmid-free derivative of NCDO 712	13
<i>B. subtilis</i> 168	<i>trpC2</i>	28
ATCC 6633	Subtilin producer	11
ATCC 6633 <i>spaB</i>	<i>spaB</i> ::Cm <sup>r</sup>	24
NZ8900	168, <i>amyE</i> :: <i>spaRK</i> , Km <sup>r</sup>	This study
NZ8901	168, <i>amyE</i> ::Km <sup>r</sup>	This study
<b>Plasmids</b>		
pBTK2	<i>amyE-lacZ</i> insertion vector, PE1 derivative, Ap <sup>r</sup> Km <sup>r</sup>	34
pNZ8032	NICE expression vector, $P_{nisA}$ , Cm <sup>r</sup>	10
pNZ8900	<i>amyE-spaRK, lacZ</i> insertion vector, PE1 derivative, Amp <sup>r</sup> Km <sup>r</sup>	This study
pNZ8901	SURE expression vector, $P_{spaSmut}$ , Cm <sup>r</sup>	This study
pNZ8902	SURE expression vector, $P_{spaSmut}$ , Em <sup>r</sup>	This study
pNZ8903	$P_{spaS}$ translationally fused to <i>gusA</i> , Em <sup>r</sup>	This study
pNZ8904	$P_{spaS}$ translationally fused to <i>gusA</i> , Cm <sup>r</sup>	This study
pNZ8905	$P_{spaSmut}$ translationally fused to <i>gusA</i> , Em <sup>r</sup>	This study
pNZ8906	$P_{spaSmut}$ translationally fused to <i>gusA</i> , Cm <sup>r</sup>	This study
pNZ8907	$P_{spaS}$ translationally fused to <i>gfp</i> , Em <sup>r</sup>	This study
pNZ8908	$P_{spaSmut}$ translationally fused to <i>gfp</i> , Em <sup>r</sup>	This study
pNZ8910	SURE expression vector, $P_{spaS}$ , Em <sup>r</sup>	This study
pNZ8911	SURE expression vector, $P_{spaS}$ , Cm <sup>r</sup>	This study
pUC19Ery	<i>lacZ</i> , Ap <sup>r</sup> Em <sup>r</sup>	26

heterologous intracellular reporters  $\beta$ -glucuronidase (GusA) and green fluorescent protein (GFP). The results show that the SURE system provides a strictly controlled and effective gene expression toolbox for *B. subtilis*.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* MC1061 (6) and *L. lactis* MG1363 (13) were used as intermediate cloning hosts. *B. subtilis* and *E. coli* were grown aerobically at 37°C in TY medium (19). Minimal medium for *B. subtilis* was prepared as described previously (47). When appropriate, *B. subtilis* growth media were supplemented with chloramphenicol (5  $\mu$ g/ml), erythromycin (5  $\mu$ g/ml), or kanamycin (10  $\mu$ g/ml), and *E. coli* growth media were supplemented with chloramphenicol (10  $\mu$ g/ml), erythromycin (150  $\mu$ g/ml), or ampicillin (50  $\mu$ g/ml). *L. lactis* was grown at 30°C in M17 broth (Oxoid, Basingstoke, England) supplemented with 0.5% (wt/vol) glucose, to which erythromycin (10  $\mu$ g/ml) was added when appropriate.

**Recombinant DNA techniques.** Plasmid DNA was isolated from *E. coli* as previously described (3) and was purified by anion-exchange chromatography on JetStar columns (Genomed, Oberhausen, Germany). Procedures for DNA manipulation and transformation of *E. coli* were carried out as described by Sambrook et al. (40). Enzymes were obtained from Roche (Mannheim, Germany), Amersham Pharmacia Biotech (Roosendaal, The Netherlands), or Gibco BRL

TABLE 2. Primers used in this study

Primer	Sequence (5' to 3') <sup>a</sup>	Description and location
PspaR-F	CGCAGGATCCGCATGAAATAAATTCAGGGGTATTG	BamHI, upstream of P <sub>spaR</sub>
spaK-R	CGAAAGAGCTTCTAGAGAAGATGGATCAG	XbaI, downstream of <i>spaK</i>
PspaS-F	GCCAAGATCTTAAAAAAGGAAAAAATGATAAAATCTTG	BglII, upstream of P <sub>spaS</sub>
PspaSmut-F	GCCAAGATCTTAAAAAAGGAAAAAATGATAAAATCTTG <sup>b</sup>	BglII, upstream of P <sub>spaS</sub>
PspaS-R	CGCACCATGGTGGTGCACCTCCTTTCAATACC	NcoI, 5' end of <i>spaS</i>
gfp-F	GCGCTCATGAGTAAAGGAGAAGAACTTTTCACTGG	RcaI, 5' end of <i>gfpmut1</i>
gfp-R	GCCCAAGCTTATTATTTGTAGAGCTCATCCATGCCATGTG	HindIII, 3' end of <i>gfpmut1</i>

<sup>a</sup> Relevant restriction sites are underlined.

<sup>b</sup> The mutation in the *spaS* promoter is indicated by italics.

Life Technologies (Breda, The Netherlands) and were used according to the manufacturers' protocols. *B. subtilis* was transformed as described previously (47). The primers (Prologo, Paris, France) used in this study are listed in Table 2. PCR was performed using *Taq* DNA polymerase (Amersham Pharmacia Biotech, Roosendaal, The Netherlands; Gibco BRL Life Technologies, Breda, The Netherlands) or *Pwo* DNA polymerase (Roche Diagnostics, Mannheim, Germany) and a DNA thermocycler (Perkin-Elmer, Shelton, Conn.). The anticipated sequences of all cloned PCR products were confirmed by DNA sequence analysis (BaseClear, Leiden, The Netherlands).

**Plasmid and strain construction.** To introduce the *spaRK* genes into the *B. subtilis* chromosome, these genes, including their promoter, were amplified by PCR using primers *spaR*-F and *spaK*-R and chromosomal DNA of *B. subtilis* ATCC 6633 (11) as the template. After digestion of the PCR product with BamHI and XbaI, the resulting 2.2-kb *spaRK* fragment was cloned between the *amy*-front and *amy*-back homologous fragments of similarly digested pBTK2 (34), yielding pNZ8900. This *spaRK* integration plasmid was transformed into *B. subtilis* 168, and kanamycin-resistant candidate integrants were analyzed for a lack of halos on plates containing 1% starch to confirm that there was correct double-crossover integration at the *amyE* locus. A Km<sup>r</sup>, *amyE* single-colony isolate was designated NZ8900. As a reference, *B. subtilis* 168 was transformed with the original integration vector, pBTK2, by using the same procedure. The resulting Km<sup>r</sup>, *amyE* colony isolated was designated NZ8901 (Table 1).

To construct plasmids harboring the *spaS* promoter and its mutant derivative that were translationally fused to the *gusA* reporter gene, both promoter fragments were amplified by PCR using primers PspaS-F and PspaS-R (wild-type *spaS* promoter) and primers PspaSmut-F and PspaS-R (mutated *spaS* promoter) and chromosomal DNA of *B. subtilis* ATCC 6633 as the template. The mutation introduced into the mutated *spaS* promoter resulted in the presence of a perfect consensus pentanucleotide repeat (TTGAT) in the *spa* box of this promoter (22, 44). After digestion of the resulting 80-bp PCR products with BglII and NcoI, the promoter regions were ligated into the corresponding sites of pNZ8032 (10). Plasmids harboring the wild-type and mutated *spaS* promoter fragments were designated pNZ8904 and pNZ8906, respectively (Table 1). Erythromycin-resistant variants of these plasmids were constructed by removal of the chloramphenicol resistance gene by digesting the plasmids with StuI and SalI. The 3.8-kb vector fragments were ligated to the 1.1-kb erythromycin resistance cassette which was obtained as a HindIII-XbaI fragment from pUC19Ery (26) after Klenow filling of the cohesive ends of both the vector and the insert. The resulting plasmids were designated pNZ8903 (P<sub>spaS</sub>) and pNZ8905 (P<sub>spaSmut</sub>) (Table 1).

To translationally fuse the *gfpmut1* gene with either the wild-type *spaS* promoter region or the mutated *spaS* promoter, this gene was amplified using primers gfp-F and gfp-R (Table 2) and chromosomal DNA of *B. subtilis* strain IIA-gfp (46) as the template. The 720-bp amplicon obtained was cloned into SmaI-digested pUC18 (40). The *gfpmut1* gene was recovered from the resulting plasmid as an RcaI-HindIII fragment and cloned in NcoI-HindIII-digested pNZ8903 and pNZ8905, resulting in pNZ8907 and pNZ8908, respectively (Table 1).

To construct convenient SURE cloning vectors, the 700-bp NcoI-Eco47III fragment of pNZ8048 (10), which contained a multiple cloning site and a transcription terminator, was cloned into similarly digested pNZ8903, pNZ8904, pNZ8905, and pNZ8906, resulting in plasmids pNZ8901, pNZ8902, pNZ8910, and pNZ8911 (Table 1).

**Activation of the *spaS* promoters by subtilin and enzymatic analysis.** For subtilin-mediated induction of gene expression, a fresh overnight culture of the appropriate *B. subtilis* strain was inoculated into fresh medium at an optical density at 600 nm (OD<sub>600</sub>) of 0.15. When cultures reached an OD<sub>600</sub> of 1.0, they were split into portions, and a range of concentrations (0 to 10%, vol/vol) of

subtilin-containing culture supernatant of *B. subtilis* ATCC 6633 was added to induce gene expression. To do this, fresh overnight culture supernatants of *B. subtilis* ATCC 6633 were heated for 10 min at 80°C to eliminate residual living *B. subtilis* ATCC 6633 cells. Following induction, cultures were grown for another 2 h prior to quantitative analysis of the reporter proteins. For determination of β-glucuronidase activity, cells were harvested by centrifugation (10 min, 6,000 × g, 4°C), washed, and resuspended in a 50 mM sodium phosphate buffer (pH 7.0) at an OD<sub>600</sub> of 10. Cell extracts were prepared by bead beating (33), and the amount of GusA was determined spectrophotometrically by using *para*-nitrophenyl-β-D-glucuronic acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) (9). Specific activities were calculated based on the amount of total protein determined as described by Bradford (4). Overproduction of GusA protein was visualized by Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29, 32).

**Fluorescence microscopy and flow cytometry.** Cells were prepared for microscopy and applied to agarose slides as described previously (47). Fluorescent signals of GFP were visualized using set 09 (excitation at 450 to 490 nm and emission at >520 nm), and images were acquired using an Axiophot microscope equipped with an AxioVision camera (Zeiss, Oberkochen, Germany). The AxioVs20 software (Zeiss, Oberkochen, Germany) was used for image capturing, and figures were prepared using Corel Graphics Suite 11 (Corel Corporation, Unterschleissheim, Germany). Flow cytometry measurements were obtained as described previously (46).

## RESULTS

Based on the strong parallel between nisin regulation mechanisms and subtilin regulation mechanisms, we decided to evaluate the characteristics of a subtilin-regulated expression system for controlled gene expression in *B. subtilis*. Previous studies of the strengths of the *spaS*, *spaB*, and *spaI* promoters have shown that the *spaS* promoter drives the highest level of transcription activation upon induction with subtilin (22, 44). Based on this observation and taking into account the fact that the P<sub>spaS</sub> *spa* box contains one mismatch compared to *spa* boxes found in P<sub>spaB</sub> and P<sub>spaI</sub> (22, 44), expression vectors containing either the wild-type *spaS* promoter or a mutant derivative in which the *spa* box had been changed into a perfect pentanucleotide direct repeat (TTGAT) were constructed, and their transcriptional activation upon subtilin induction was analyzed. To generate a production host, we introduced the subtilin sensor-regulator couple *spaRK* by integrating plasmid pNZ8900 into the *amyE* locus of *B. subtilis* 168, resulting in strain NZ8900 (Table 1).

**Subtilin-induced production of the *E. coli* β-glucuronidase reporter.** Both the wild-type *spaS* promoter and the mutant *spaS* promoter were translationally fused to the intracellularly produced β-glucuronidase gene (*gusA*) originating from *E. coli* (38). The plasmids that were constructed harbored either the chloramphenicol resistance gene (pNZ8904 and pNZ8906, respectively) or the erythromycin resistance gene (pNZ8903 and pNZ8905, respectively) (Table 1) and were transformed into



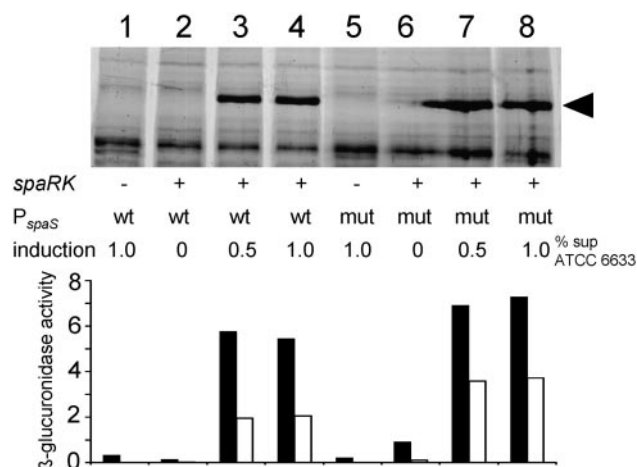


FIG. 1. Subtilin-regulated overexpression of  $\beta$ -glucuronidase (GusA) in *B. subtilis*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining was performed with crude cell extracts of *B. subtilis* NZ8900 harboring plasmids pNZ8904 ( $P_{spaS}$ -gusA) (lanes 3 to 5) and pNZ8906 ( $P_{spaSmut}$ -gusA) (lanes 6 to 8) that were not induced and were induced with 0.5 and 1.0% (vol/vol) culture supernatant of *B. subtilis* ATCC 6633 (lanes 2 and 6, 3 and 7, and 5 and 8, respectively). The control samples were *B. subtilis* NZ8901 harboring plasmids pNZ8904 and pNZ8906 after induction with 1% (vol/vol) culture supernatant of *B. subtilis* ATCC 6633 (lanes 1 and 5, respectively). The specific activities (in  $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ ) of GusA in *B. subtilis* NZ8900 or NZ8901 (solid bars) and *B. subtilis* ATCC 6633 *spaB* harboring pNZ8903 or pNZ8905 (open bars) are also shown. The data are from a representative induction experiment.

the *spaRK*-expressing strain *B. subtilis* NZ8900 (pNZ8904 and pNZ8906) or into the chloramphenicol-resistant *spaB* mutant of *B. subtilis* ATCC 6633 (pNZ8903 and pNZ8905). The latter strain is a *spaB* mutant derivative of the subtilin-producing strain ATCC 6633, which contains all genes involved in subtilin autoinduction and biosynthesis but does not produce subtilin due to the *spaB* mutation (24) (Table 1). The specific activities of GusA revealed that both *spaS* promoter variants were functional for driving GusA activity in both *B. subtilis* NZ8900 and strain ATCC 6633 *spaB* after induction with the subtilin-containing supernatant of *B. subtilis* ATCC 6633 (Fig. 1). The consensus *spa* box present in the mutant *spaS* promoter resulted in higher GusA activity in both *spaRK*-containing strains compared to the activity observed with the wild-type *spaS* promoter (Fig. 1, compare lanes 7 and 8 with lanes 3 and 4), which confirmed the “optimized” characteristics of this mutant *spaS* promoter. However, the GusA activity that was driven from

the mutant *spaS* promoter under noninducing conditions appeared to be greater than the GusA activity obtained with the wild-type *spaS* promoter (Fig. 1, lanes 6 and 2, respectively). This leakage of the mutant *spaS* promoter appeared to be *spaRK* dependent since the background GusA activity levels in strains that lacked *spaRK* appeared to be similar to those obtained with the cloning vector alone (Fig. 1, compare lane 6 with lanes 1 and 5). Moreover, for both promoter-fusion constructs no significant increase in  $\beta$ -glucuronidase activity was observed upon induction with 1.0% (vol/vol) supernatant of *B. subtilis* ATCC 6633 compared to induction with 0.5% (vol/vol) supernatant of *B. subtilis* ATCC 6633 (Fig. 1, compare lanes 4 and 8 with lanes 3 and 7, respectively). This suggests that the maximal level of induction was achieved upon induction with 0.5% (vol/vol) supernatant of *B. subtilis* ATCC 6633. The production of GusA driven by both  $P_{spaS}$  variants appeared higher in the *spaRK* integrant strain NZ8900 than in the *spaB* derivative of strain ATCC 6633 (Fig. 1), which could have been related to higher *spaRK* expression in NZ8900.

**Subtilin-induced production of the GFP reporter.** To evaluate the tightness of expression control and the dynamic range of both promoter variants in more detail, flow cytometric measurements using GFP as a reporter were obtained. This reporter is known to exhibit high sensitivity and has an extended linear dynamic range (8, 39). *B. subtilis* NZ8900 harboring plasmids pNZ8907 and pNZ8908, which contained  $P_{spaS}$ -gfp and  $P_{spaSmut}$ -gfp translational fusions, respectively (Table 1), were subjected to induction with a range of concentrations of culture supernatant of strain ATCC 6633. *B. subtilis* NZ8900 harboring pNZ8902 was used as a control. Fluorescent microscopy revealed that GFP production driven from the wild-type and mutant *spaS* promoters in *B. subtilis* NZ8900 was observed in all individual cells upon induction with 0.5% (vol/vol) supernatant of *B. subtilis* ATCC 6633 (Fig. 2B and C, respectively). In contrast, without subtilin induction no fluorescence was observed for either the wild-type *spaS* promoter (data not shown) or the mutant *spaS* promoter (Fig. 2A). As anticipated, cells harboring the control plasmid pNZ8902 displayed no fluorescence upon induction with 0.5% (vol/vol) supernatant of *B. subtilis* ATCC 6633 (data not shown). To quantitatively assess wild-type and mutant *spaS* promoter activities, the mean fluorescence intensities obtained by flow cytometry were calculated relative to the mean fluorescence of the  $P_{spaSmut}$ -gfp construct upon induction with 0.2% (vol/vol) supernatant of *B. subtilis* ATCC 6633 (Table 3). The results obtained clearly established

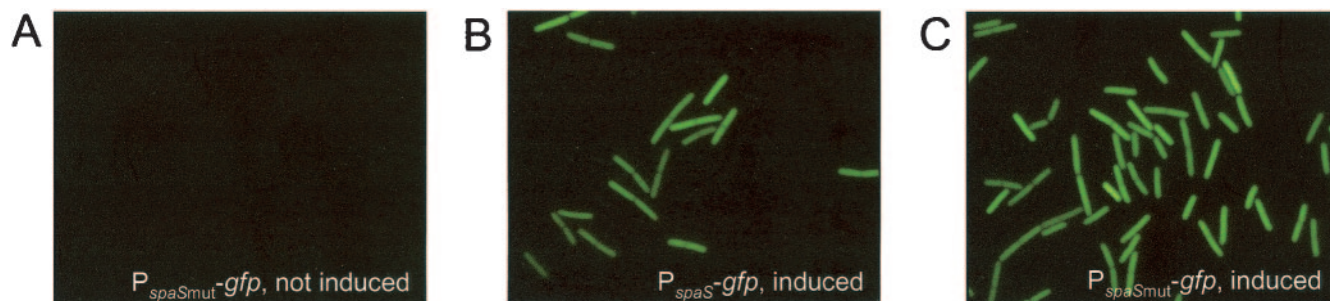


FIG. 2. Fluorescence microscopy of subtilin-induced GFP production in *B. subtilis*: representative views of *B. subtilis* NZ8900 harboring pNZ8908 ( $P_{spaSmut}$ -gfp) without and with subtilin induction (A and C, respectively) and pNZ8907 ( $P_{spaS}$ -gfp) with subtilin induction (B). In all cases when subtilin induction was used, 0.5% (vol/vol) *B. subtilis* ATCC 6633 supernatant was used.

TABLE 3. Dynamics of the wild-type and mutated *spaS* promoters<sup>a</sup>

Construct	Mean fluorescence (%) with <sup>b</sup> :			
	No supernatant	0.1% (vol/vol) supernatant	0.2% (vol/vol) supernatant	0.5% (vol/vol) supernatant <sup>c</sup>
Empty vector	0	0	0	0
P <sub><i>spaS</i></sub>	0.5	14.5	42.8	57.3
P <sub><i>spaSmut</i></sub>	1.2	65.0	100	98.4

<sup>a</sup> Cultures of *B. subtilis* NZ8900 harboring pNZ8902 (empty plasmid), pNZ8908 (P<sub>*spaSmut*</sub>-gfp), or pNZ8907 (P<sub>*spaS*</sub>-gfp) were grown to an OD<sub>600</sub> of 1, and the supernatant of an overnight culture of subtilin-producing strain ATCC 6633 was added at the concentrations indicated. After 2 h, cells were collected for flow cytometric analyses.

<sup>b</sup> Each value is the percentage of the mean fluorescence of the population compared to the maximal observed fluorescence. This value was calculated as follows: the mean fluorescence of 20,000 cells (calculated using WinMDI [http://facs.scripps.edu/software.html]) was divided by the observed mean fluorescence after induction with 0.2% (vol/vol) supernatant of *B. subtilis* ATCC 6633 of the construct with the P<sub>*spaSmut*</sub> promoter, normalized to the empty vector, and multiplied by 100%. The results of a representative experiment are shown.

<sup>c</sup> No significant increase in fluorescence intensity was observed upon induction with more than 0.5% (vol/vol) culture supernatant of *B. subtilis* ATCC 6633.

that the P<sub>*spaSmut*</sub>-derived level of expression at a fixed inducer concentration was higher than the level generated by the wild-type *spaS* promoter (Table 3 and Fig. 3D). Notably, the mutant *spaS* promoter also generated maximal levels of induction with lower levels of inducer (Table 3). The leakage under nonin-

ducing conditions appeared to be extremely low for both promoter variants. Nevertheless, the wild-type *spaS* promoter exhibited lower levels of basal GFP production than the mutated *spaS* promoter exhibited, which is analogous to what was observed with the GusA reporter (Table 3 and Fig. 3C). Overall, these quantitative GFP measurements established that the wild-type *spaS* promoter activity displayed at least a 110-fold increase in activity upon expression, while its (“optimized”) mutant derivative could be induced at least 80-fold when the maximal induced/uninduced ratio was calculated from the data in Table 3. Moreover, the single-cell GFP analyses clearly showed that induced cultures displayed a homogeneous response to subtilin induction and confirmed the lack of culture heterogeneity indicated by fluorescence microscopy.

# DISCUSSION

The β-glucuronidase (GusA) and GFP reporters used to determine the characteristics of the SURE system in *B. subtilis* established that wild-type *spaS* promoter activity is controlled more strictly by the inducer subtilin than its *spa* box consensus derivative is. However, the latter promoter generated higher levels of expression under maximal induction conditions. The relative expression levels could be increased at least 110-fold and 80-fold using the wild type and the mutant derivative of the

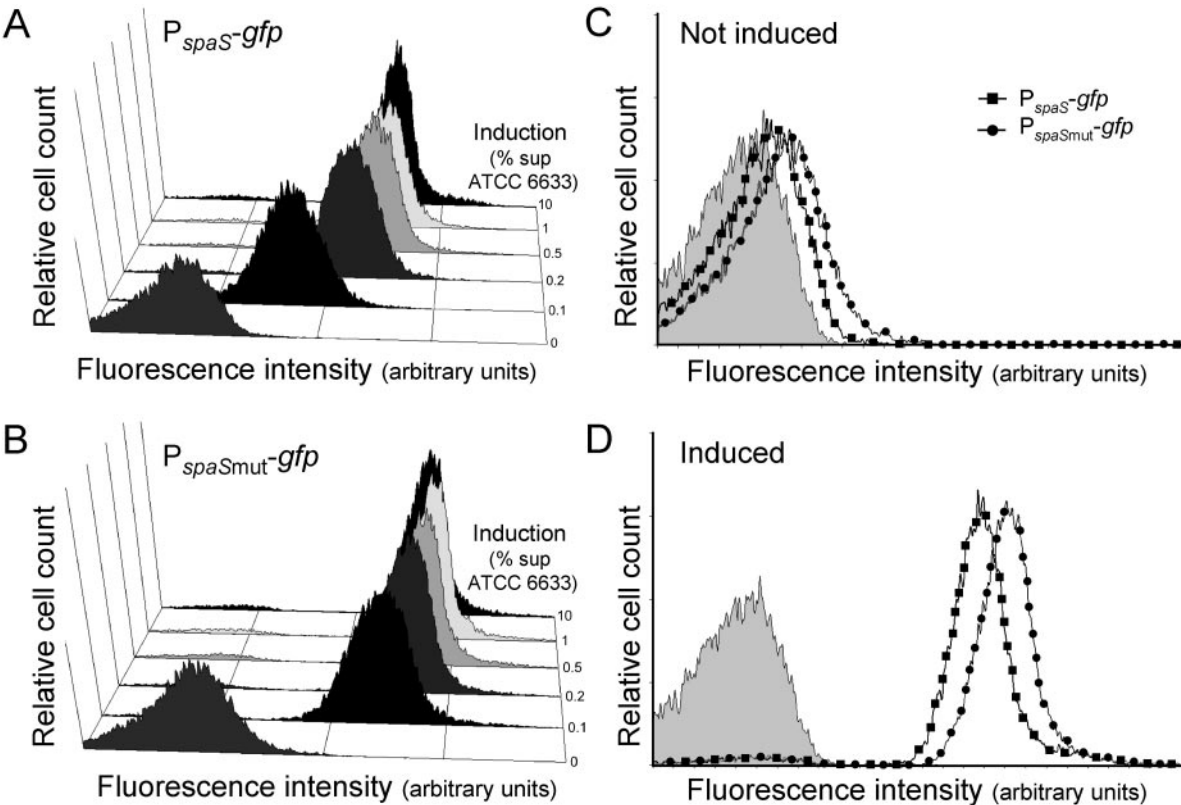


FIG. 3. Subtilin-regulated overexpression of GFP in *B. subtilis*: relative numbers of cells (z axis) displaying fluorescence intensities measured by flow cytometry of GFP, indicated in arbitrary units (x axis) for *B. subtilis* NZ8900 harboring pNZ8907 (A) and pNZ8908 (B) without induction and with induction with 0.1, 0.2, 0.5, 1.0, and 10% (vol/vol) supernatant of *B. subtilis* ATCC 6633. The tightness (without induction) (C) and the activity with maximal induction (10% [vol/vol] supernatant of *B. subtilis* ATCC 6633) (D) are shown for *B. subtilis* NZ8900 carrying the empty vector pNZ8902 (shaded area), the P<sub>*spaS*</sub>-gfp fusion pNZ8907 (■), or the P<sub>*spaSmut*</sub>-gfp fusion pNZ8908 (●). The symbols are used to distinguish the lines; however, they do not represent measuring points.

*spaS* promoter upon subtilin-mediated induction, respectively. Since the desired expression characteristics strongly depend on the nature of the gene and the corresponding function that is expressed, both promoter variants could be useful. Therefore, a range of SURE expression vectors in which the gene of interest could be translationally or transcriptionally fused to the different *spaS* promoters were constructed (Table 1).

The *amyE* locus targeted for integration of the genes encoding the subtilin-responsive regulatory module *spaRK* appears to be well conserved among *B. subtilis* strains, indicating that the same integration vector can be used to implement the SURE system in alternative *B. subtilis* backgrounds. An example of this broad application range is the successful and functional integration of *spaRK* in various *B. subtilis* strains whose proteolytic capacities are affected, which is an attractive background for the production of secreted proteins and enzymes (data not shown). This provides a clear parallel with the *L. lactis* NICE system, in which chromosomal integrations of *nisRK* have been obtained using the *pepN* locus as the site of integration (27). Based on the similarity between the regulatory characteristics of the NICE and SURE systems, it can be expected that the SURE system in *B. subtilis* will allow similar possibilities in both fundamental and industrial research applications, including controlled overexpression of a variety of homologous and heterologous proteins and enzymes. Additionally, and in analogy with the NICE system, the strict control and lack of significant leakage of the SURE system under noninducing conditions also provide possibilities for controlled production of toxic gene products or construction of conditional mutations in essential genes in *B. subtilis* (for a recent review of the NICE application potential see reference 35). An advantage of *B. subtilis* over *L. lactis* is the high cell densities that can be obtained readily using the former host in industrial production, while the fermentative characteristics of *L. lactis* prevent its growth to high cell densities under normal industrial conditions. Thus, *B. subtilis* would most likely be the production host of choice for high-yield gram-positive bacterial protein production on an industrial scale. Moreover, *B. subtilis* is well known as a convenient host for production of secreted proteins, and the secretion capacities of this species have been studied in detail (5).

In *L. lactis* the nisin regulatory genes, *nisRK*, are constitutively expressed (9). In contrast, in *B. subtilis* production of subtilin has been reported to be dually controlled since expression of *spaRK* was shown to be growth phase dependent due to sigma H-dependent *spaR* promoter control (43). This cell-density-dependent transcription of *spaR* and *spaK* apparently did not affect our observations, since high levels of production of the reporters were observed during the early log and mid-log growth phases.

Various growth-phase-dependent phenotypes in *B. subtilis* appear to be subpopulation responses rather than homogeneous culture responses (42, 46). The most prominent example of this differentiation is probably the development of competence in this species, where only approximately 10% of all cells in a culture of *B. subtilis* develop the competence phenotype (15, 16). In analogy, it could be that the growth-phase-dependent production of subtilin is also restricted to subpopulations of *B. subtilis* cultures. The results presented here, especially the flow cytometric single-cell measurements (Fig. 3), show that

expression of the *spaS* promoter upon activation via subtilin induction is homogeneous, as indicated by a single sharp peak of fluorescence for the GFP-expressing population. This shows that all cells in the responsive *B. subtilis* culture respond in a similar way to the extracellular inducer subtilin, which is to be expected in a continuously shaken liquid culture.

Overall, the characteristics of the SURE system enable strictly controlled, high-level expression of a gene of interest, and thus this system is a novel, easy-to-handle, and robust expression system for *B. subtilis*. At this stage, comparisons of the SURE system with alternative controlled expression systems available for this species (for example, the  $P_{spac}$  and  $P_{xyLA}$  systems) can be done only in an indirect manner. Many technical aspects of the previously described studies were significantly different from those described here. For example, the SURE system employs plasmid-based gene expression, while the  $P_{spac}$  and  $P_{xyLA}$  systems in many cases depend on chromosomal integration of the promoter and the gene of interest. The consequences of such copy number or gene dosage differences are difficult to predict, but the differences certainly illustrate the strictness of gene expression control (lack of leakage) of the SURE system. In addition, changing the plasmid vector and corresponding gene dosage should allow further fine-tuning of the SURE system. Another major advantage of the SURE system compared to the  $P_{spac}$  and  $P_{xyLA}$  systems is based on the fact that the SURE system is not derived from sugar-fermenting capacities of *B. subtilis*. Thus, the SURE system lacks the glucose repression control observed for the  $P_{spac}$  and  $P_{xyLA}$  systems (2), which avoids the need to alter the medium composition prior to induction of gene expression that is required to obtain strict control in the latter two systems. Furthermore, the SURE system can be regarded as a self-cloning product for *B. subtilis* and has several advantages over implementation of the NICE system in this host, as follows: (i) implementation of the NICE system has been achieved via a dual-plasmid system (12), while the SURE system depends only on a single plasmid and is expected to generate a more stable expression platform; (ii) the maximal *gusA* expression levels achieved in *B. subtilis* using the SURE system are significantly higher than those observed for the NICE system; and (iii) the maximal *gusA* expression levels are achieved at a much lower concentration of the inducer molecule. The latter fact could be especially relevant in view of the antimicrobial activity of the inducer at higher concentrations. Overall, our results show that the SURE system drives extremely high levels of gene expression, while its leakage is virtually undetectable. Finally, based on our and colleagues' experiences with the other inducible systems, we feel that this system may have the most desirable characteristics known among the available systems for inducible gene expression in *B. subtilis*.

In conclusion, the SURE system described here adds a valuable tool for genetic engineering in *B. subtilis*. The availability of various expression vectors with slightly different expression characteristics ( $P_{spaS}$  versus its *spa* box consensus derivative) allows a "designer" approach toward the construction of expression systems for individual genes. Moreover, the effective transfer of the SURE system to alternative *B. subtilis* production hosts enables production of a protein of choice in a genetic background of choice.



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